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IP-10 Is Critical for Effector T Cell Trafficking and Host Survival in *Toxoplasma gondii* Infection

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Summary

The generation of an adaptive immune response against intracellular pathogens requires the recruitment of effector T cells to sites of infection. Here we show that the chemokine IP-10, a specific chemoattractant for activated T cells, controls this process in mice naturally infected with *Toxoplasma gondii*. Neutralization of IP-10 in infected mice inhibited the massive influx of T cells into tissues and impaired antigen-specific T cell effector functions. This resulted in >1000-fold increase in tissue parasite burden and a marked increase in mortality compared to control antibody–treated mice. These observations suggest that IP-10 may play a broader role in the localization and function of effector T cells at sites of Th1 inflammation.

Introduction

The generation of an adaptive immune response involves the highly regulated trafficking of T cells, B cells, and dendritic cells. Dendritic cells pick up antigen in the tissue and then migrate to regional lymph nodes, where they encounter and activate naive T and B cells (Banchereau and Steinman, 1998). These activated effector lymphocytes leave the lymph node and enter the bloodstream, and must then find their way to the site where foreign antigen was originally encountered. Recent studies have demonstrated that specific chemokines control the movement of naive T and B cells from the blood and antigen-loaded dendritic cells from the tissue into secondary lymphoid tissue to initiate an adaptive immune response (Cyster, 1999). However, the specific chemokines that guide the migration of activated effector T cells from secondary lymphoid tissues into sites of infection and inflammation remain ill-defined.

In vitro studies examining the expression and function of chemokine receptors on activated T cells have suggested that specific chemokines may control polarized $CD4^+$ T cell trafficking. Activated Th1 cells express CXCR3 and CCR5 and respond to the CXCR3 ligands IP-10 (interferon-induced protein of 10 kDa), Mig (monokine induced by gamma-interferon), and I-TAC (interferon-inducible T cell α chemoattractant) and the CCR5 ligands MIP-1α (macrophage inflammatory protein-1α), MIP-1β, and RANTES (Bonecchi et al., 1998; Loetscher et al., 1998; Sallusto et al., 1998). These in vitro studies have been supported by studies examining chemokine and chemokine receptor expression in diseased tissue. Specific disease states for which Th1-type inflammatory responses predominate include atherosclerosis, sarcoidosis, rheumatoid arthritis, and viral hepatitis. We have recently reported that human atheroma express high levels of IP-10, Mig, and I-TAC and that virtually all of the T cells found in atheromatous lesions were CXCR3 positive (Mach et al., 1999). Likewise, high levels of IP-10 were found in the broncho-alveolar lavage (BAL) fluid of patients with active pulmonary sarcoidosis and all of the BAL T cells were CXCR3 positive (Agostini et al., 1998). Similarly, in rheumatoid arthritis patients, T cells recovered from synovial fluid expressed higher levels of CXCR3 and CCR5 than peripheral blood T cells (Qin et al., 1998). Recently, it has been reported that lymphocytes infiltrating the liver of patients with chronic hepatitis C expressed CXCR3 and CCR5 (Shields et al., 1999). Interestingly, whereas the CCR5 ligands MIP-1α and MIP-1β were confined to the vessels within the portal tract, the CXCR3 ligands IP-10 and Mig were selectively upregulated on sinusoidal endothelium. These findings suggest a specific role for CXCR3 ligands, as opposed to CCR5 ligands, in the recruitment of T cells into the hepatic parenchyma. Taken together, the above studies implicate IP-10, Mig, and I-TAC in regulating Th1 cell trafficking into tissue where there is ongoing Th1 inflammation. However, the functional importance of these chemokines and their receptor CXCR3 in Th1-type inflammation remains to be experimentally tested, which was the goal of the present study.

An established murine model of *Toxoplasma gondii* infection was used to study the role of chemokines in generating organ-specific adaptive Th1-type immunity. Natural infection with *T. gondii* results from the ingestion of cysts or oocytes found in infected animal meat or cat feces, respectively. Once ingested, the parasite matures into a tachyzoite, which then invades the intestinal tract and disseminates to most organs. *T. gondii* induces a strong cell-mediated response that limits parasite growth in the tissue and leads to the transformation of the parasite from the tachyzoite into a dormant cyst, resulting in an asymptomatic chronic infection (Frenkel, 1988). This highly effective immunity is mediated by both $CD4^+$ and $CD8^+$ T cells (Suzuki and Remington, 1988; Gazzinelli et al., 1991; Gazzinelli et al., 1992; Khan et al., 1994) and is critically dependent on the Th1 cytokine IFNγ (Suzuki et al., 1988; Deckert-Schluter et al., 1996). Impairment of cell-mediated immunity, as seen in AIDS patients, leads to a more aggressive acute infection as well as cyst reactivation of chronic latent disease and fulminant reemergence of acute disease (Luft et al., 1984).

We analyzed the expression of multiple chemokines in infected tissue and found the preferential induction

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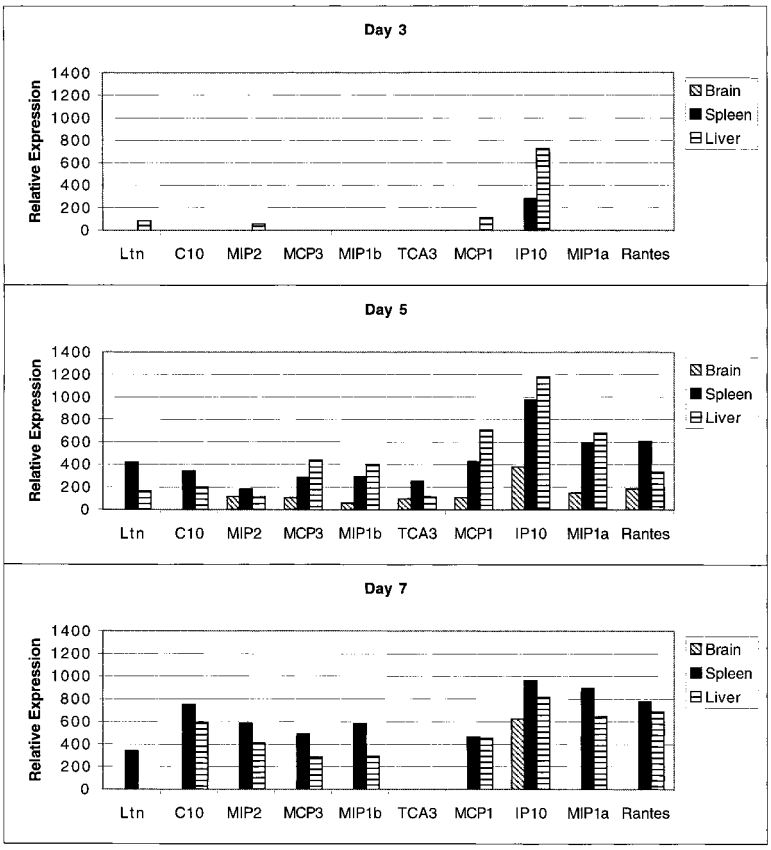

Figure 1. Chemokine Response in *T. gondii*–Infected Mice

The expression of chemokine mRNA in the brain, spleen, and liver of C57BL/6 mice (female, 5–6 weeks old) infected orally with 15 cysts of 76K strain of *Toxoplasma gondii*. RNase protection analysis was performed on total RNA isolated from infected animals on days 0, 3, 5, and 7 post infection. RNA was pooled from three mice for each organ, and each time point studied. The murine chemokines studied included Ltn (lymphotactin), C10, MIP-2 (macrophage inflammatory protein-2), MCP-3 (macrophage chemoattractant protein-3), MIP-1β, MCP-1, IP-10, MIP-1α, and RANTES. Expression of each chemokine was normalized to the expression of the control ribosomal protein gene rpL32.

of IP-10 at early time points in infected tissue. Since IP-10 is profoundly induced in cells by IFNγ (Luster et al., 1985; Luster and Ravetch, 1987) and IFNγ is critical for host defense against *T. gondii* (Suzuki et al., 1988), we investigated the role of IP-10 in the host response to acute toxoplasma infection. To accomplish this, we have generated neutralizing monoclonal antibodies (mAbs) to IP-10 that block its activation of CXCR3. Using these mAbs, we found that IP-10 was required for survival following *T. gondii* infection and controlled the trafficking and function of activated effector T cells in tissues.

Results

Chemokine Expression in *T. gondii* Infection

To determine the kinetics of chemokine induction post natural infection, we examined the expression of several chemokines in the tissues of mice challenged orally with cysts of *T. gondii* (Figure 1). Of the 10 chemokines examined, at early time points (days 3 and 5 post challenge) there was preferential expression of IP-10 mRNA compared to the other chemokines in all infected tissues analyzed (brain, spleen, liver, and lung) (Figure 1; data not shown). By day 7, a significant induction of multiple chemokines was noted in the spleen, liver, and lung (Figure 1; data not shown); however, IP-10 was still preferentially expressed in the brain.

We were interested in the expression pattern of the other known IFNγ-inducible CXCR3 ligands, Mig and I-TAC, that were not evaluated in the ribonuclease protection assay. The expression of IP-10, Mig, and I-TAC was therefore analyzed by Northern blot on RNA isolated from the brain, liver, lung, spleen, and gut of mice infected with *T. gondii* at 3, 7, and 14 days post infection (Figure 2). IP-10 was profoundly induced in all organs examined. In the liver, lung, and spleen, IP-10 induction was seen by day 3, while in the brain and gut, induction was not apparent until day 7. Expression of IP-10 peaked by day 7 in the gut, liver, spleen, and lung but was highest in the brain on day 14. Constitutive expression of IP-10 was seen only in the spleen and very minimally in the liver. In contrast, constitutive expression of Mig was highest in the liver and was very minimal in the spleen. Mig expression was also induced in all organs examined, although not to the same levels as IP-10. In addition, the kinetics of Mig induction was delayed compared to IP-10 and was not seen until day 7 post infection. I-TAC expression was also induced following infection but to considerably lower levels than either IP-10 or Mig and with a kinetic pattern more similar to Mig than IP-10.

Generation and Characterization of Neutralizing Anti-IP-10 mAbs

To determine the functional significance of this early and profound induction of IP-10 expression in *T. gondii*–infected tissues, we sought to neutralize IP-10 in vivo. We were particularly interested in the role of IP-10 since the host response to *T. gondii* is unaffected in mice with a targeted disruption of the Mig gene (J. Farber, personal communication). Neutralizing hamster anti-murine IP-10 mAbs were generated. 1F11 and 1B9 are IP-10 mAbs

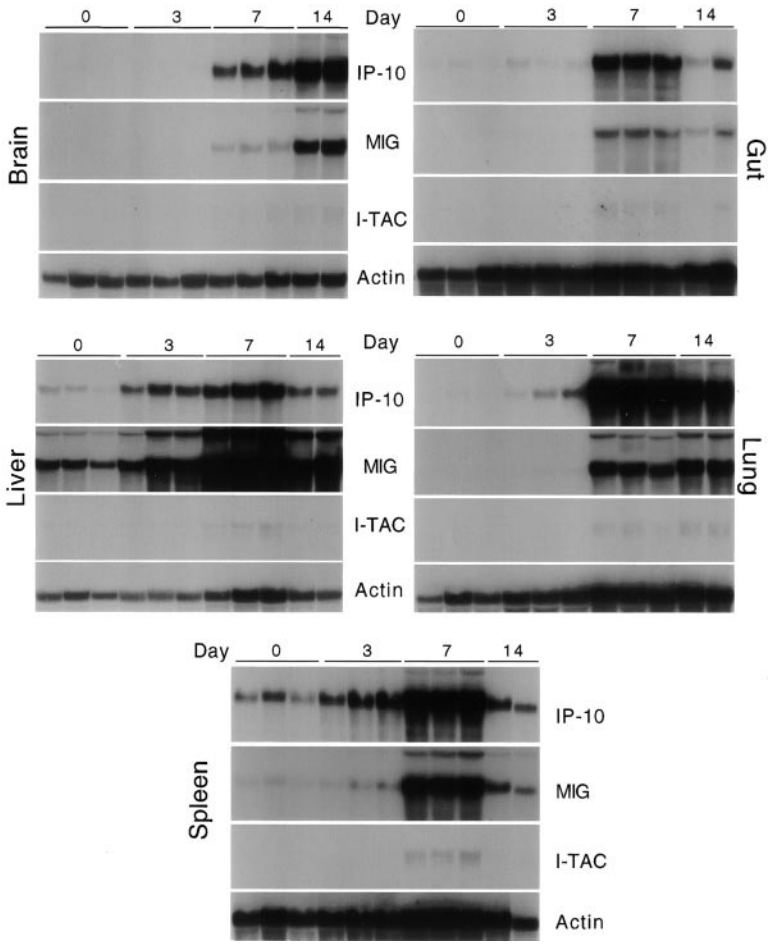

Figure 2. Expression of IP-10, Mig, and I-TAC in *T. gondii*–Infected Mice

Northern blot analysis was perfromed on total RNA isolated from the brain, spleen, liver, lung, and gut of C57BL/6 mice (female, 5–6 weeks old) infected orally with 15 cysts of 76K strain of *Toxoplasma gondii* on days 0, 3, 7, and 14 post infection. Each lane represents RNA from a different mouse. RNA was fractionated on a 1.2% agarose formaldehyde gel, transferred to GeneScreen Plus, and sequentially hybridized with ^{32}P-labeled cDNA probes for IP-10, Mig, I-TAC, and β-actin.

of differing specificity that block the ability of IP-10 to activate its receptor CXCR3 in chemotaxis and calcium flux assays (Figure 3). These mAbs are specific for murine IP-10 and did not react with other chemokines in immunoassays nor inhibit the function of other chemokines, including IP-10's closest known homolog murine Mig, with which it shares 31% amino acid identity (Figure 3). We cannot, however, at this time, exclude reactivity with murine I-TAC, which shares 26% amino acid sequence identity with murine IP-10. However, based on the limited and delayed expression of I-TAC compared to IP-10 (Figure 2), I-TAC likely plays a less significant role in the host response to *T. gondii*.

Effects of IP-10 Neutralization on the Control of *T. gondii* Infection

T. gondii–infected C57BL/6 mice treated with either anti-IP-10 mAb 1F11 or 1B9 showed 100% mortality by day 15. This was in marked contrast to the mortality seen in infected mice that received the control hamster antibody, which was <30% (Figure 4A). No ill effects were seen in uninfected mice treated with anti-IP-10 mAbs. To determine whether the increased mortality in the anti-IP-10-treated mice was accompanied by a greater parasite burden, tissues from infected mice were analyzed on days 10–12 post infection for the presence of parasite DNA. The analysis was performed by PCR, using primers for the highly conserved B1 gene (Burg et al., 1989) of *T. gondii*. Neutralization of IP-10 was associated with an ~3 log increase in tissue parasite load in the tissues of infected mice, compared to the tissues from control antibody–treated mice (Figure 4B). The markedly increased parasite burden in anti-IP-10-treated infected mice was associated with distinct histopathologic changes in liver, spleen, and intestine (Figure 4C). Livers of infected anti-IP-10-treated mice had significant signs of hepatocellular necrosis and minimal inflammation and visible tachyzoites. In contrast, livers of infected control antibody–treated mice had focal areas of mononuclear cell inflammation but minimal hepatocellular necrosis and no visible tachyzoites. The spleens of infected anti-IP-10-treated mice had areas of necrosis and tachyzoite proliferation, while spleens of infected control antibody–treated mice were devoid of necrosis and tachyzoites, consistent with resolving *T. gondii* infection. The ileal mucosa of anti-IP-10-treated infected mice had marked inflammation of the lamina propria, blunting of villi, and visible parasites, while the ileal mucosa of control antibody–treated mice had minimal inflammation and no visible tachyzoite proliferation (data not shown). Thus, neutralization of IP-10 in *T. gondii* infection resulted in a marked increase in mortality and a profound increase in tissue parasite burden, which was reflected in distinct histopathological changes in the involved organs.

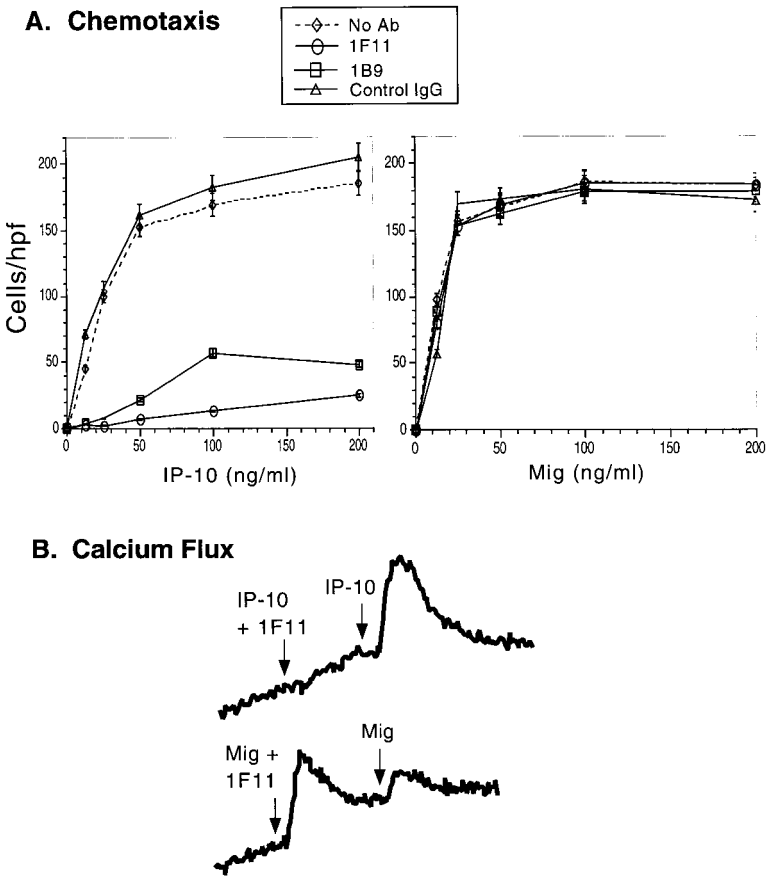

Figure 3. Anti-Murine IP-10 Monoclonal Antibodies Inhibit IP-10-Induced Chemotaxis and Calcium Flux of CXCR3-Transfected 300-19 B Cells

1F11 and 1B9 are two independent hamster mAbs specific for IP-10 with different specificities that inhibit IP-10- but not Mig-induced chemotaxis and calcium flux of murine (m) CXCR3-transfected 300-19 B cells. (A) 1F11 and 1B9 inhibit IP-10-induced chemotaxis. Increasing concentrations of murine IP-10 and murine Mig were preincubated with 200 μg of purified mAb 1F11, 1B9, or hamster IgG control prior to the addition to a modified Boyden chemotaxis chamber. The chemotactic response of mCXCR3-transfected 300-19 cells was determined by counting migrating cells per high-powered field (cells/hpf; ± SD) in eight fields from replicate wells. Data shown is a representative of four experiments. Neither IP-10 nor Mig induced chemotaxis of untransfected 300-19 cells.

(B) 1F11 and 1B9 (data not shown) inhibit IP-10-induced calcium flux. mCXCR3-transfected 300-19 cells were loaded with Fura-2, and their response to murine IP-10 (50 ng/ml) and murine Mig (50 ng/ml) preincubated with mAb (50 μg/ml) was monitored using a fluorimeter. As a control for the cells' responsiveness to CXCR3 ligands, following incubation with chemokine and mAb, free chemokine was added, which demonstrated responsiveness to IP-10. The partial desensitization seen following Mig stimulation is expected since the mAbs do not inhibit Mig-induced responses. This is a representative experiment of n = 4. Neither IP-10 nor Mig induced chemotaxis of untransfected 300-19 cells.

Effects of IP-10 Neutralization on T Cell Trafficking

To determine whether IP-10 neutralization altered lymphocyte recruitment into infected organs, we analyzed cells isolated from the spleens and livers of infected mice on days 10–12 following infection. Control antibody–treated infected mice had a substantial increase in the total lymphoid cells in both the spleen and liver, compared to uninfected mice (2.7-fold, $p < 0.05$, and 7.6-fold, $p < 0.005$, respectively) (Figures 5A and 5B). Phenotypic analysis revealed that the increased number of cells in both organs consisted mainly of $CD3^+$ lymphocytes that were of both the $CD4^+$ and $CD8^+$ phenotype. In the spleen, a smaller increase in NK cells was also observed. B220-positive cells did not change substantially in either tissue. The influx of $CD4^+$ and $CD8^+$ lymphocytes into infected spleens and livers was profoundly inhibited by neutralization of IP-10 ($p < 0.005$ and $p < 0.05$, respectively). Consistent with IP-10 mAb inhibiting the influx and subsequent expansion of T cells in infected organs, spleens (Figure 5C) and livers from anti-IP-10-treated infected mice weighed 3-fold and 1.7-fold less than the spleens and livers of control IgG–treated infected mice, respectively ($p < 0.01$ for both). IP-10 mAb and control hamster IgG treatment of uninfected control mice had no effect on the size or resident cell populations of the spleen or liver (data not shown).

To examine whether *T. gondii* infection was associated with the recruitment of cells expressing the IP-10 receptor, we examined CXCR3 expression in liver, spleen, and lung of uninfected and infected mice (Figure 6). Constitutive expression of CXCR3 was present in the spleens and very minimally in the livers and lung of uninfected mice. Control Ab–treated infected mice showed a marked increase in CXCR3 mRNA levels in the spleen, liver, and lung. In contrast, anti-IP-10 treatment completely blocked this increase in CXCR3 mRNA expression in the liver and lung and inhibited the increase in the spleen by ~40%. Anti-IP-10 treatment had no effect on CXCR3 levels in uninfected animals. These findings, taken together with the diminished recruitment of T cells into infected organs, suggest that IP-10 neutralization inhibited the recruitment of $CXCR3^+$ T cells into tissues.

Effects of IP-10 Neutralization on T Cell Effector Function

To assess whether antigen-specific immune responses generated against *T. gondii* infection were affected in anti-IP-10- and control antibody–treated mice, mitogen and antigen-specific proliferative responses were measured using fractionated $CD4^+$ and $CD8^+$ splenocytes (Figures 7A–7D). Treatment of infected mice with anti-IP-10 or control antibody had no effect on the mitogen-induced proliferation of $CD4^+$ or $CD8^+$ T splenocytes (Figures 7B and 7D). However, a significant decrease in antigen-specific proliferation was observed for both the $CD4^+$ ($p = 0.001$) and $CD8^+$ ($p = 0.003$) splenocytes

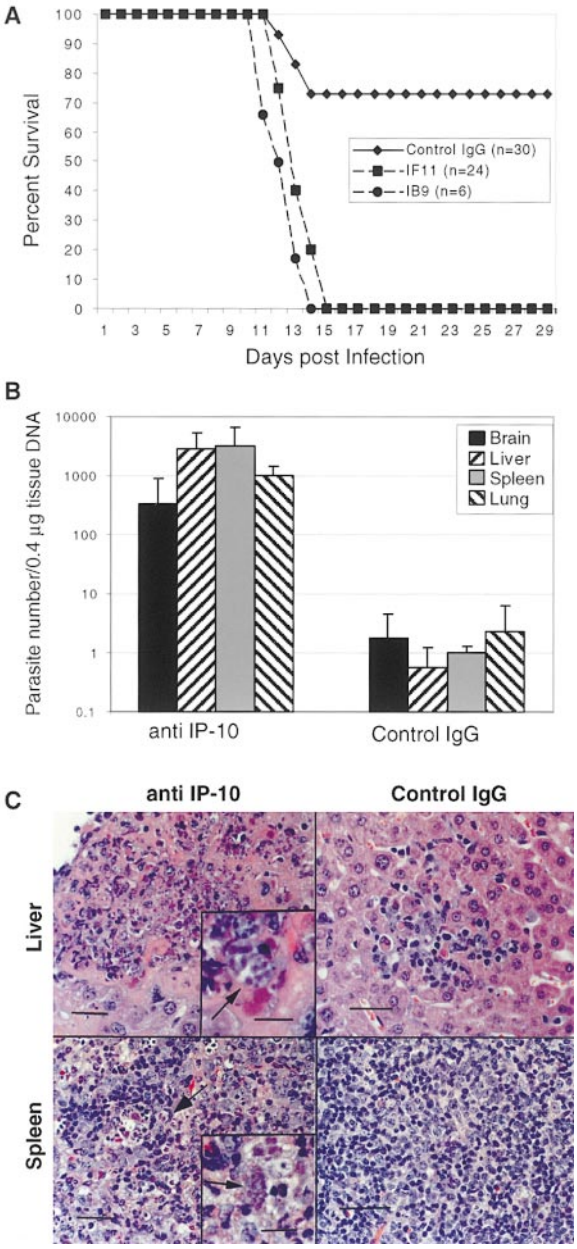

Figure 4. IP-10 Depletion Leads to Increased Mortality and Impaired Parasite Clearance Following *T. gondii* Infection in Mice

Mortality (A), tissue parasite burden (B), and histopathology (C) following oral infection with the 76K strain of *T. gondii* in anti-IP-10- and control Ab–treated C57BL/6 mice (female, 5–6 weeks old). One day prior to challenge and every other day thereafter, groups of six mice were injected i.p. with 100 μg of purified anti-IP-10 hamster mAb (1F11 or 1B9) or control hamster IgG.

(A) Cumulative percent survival for *T. gondii*–infected mice treated with 1F11 (n = 24; 4 independent experiments), 1B9 (n = 6; 1 experiment), and control IgG (n = 30; 5 experiments). Similar treatment of uninfected mice with 1F11, 1B9, or control IgG had no effects on survival.

(B) Number of parasites per microgram of tissue DNA in the brain, liver, spleen, and lung of *T. gondii*–infected animals treated with anti-IP-10 or control Ab. Groups of *T. gondii*–infected C57BL/6 mice (female, 5–6 weeks old) were treated with anti-IP-10 mAb or control IgG as described above. At days 10–12 post infection, the organs from both anti-IP-10- and control Ab–treated mice (n = 3 mice/group) were isolated, and the level of parasite load in the tissues was determined by competitive DNA PCR (Kirists et al., 2000).

(C) Photomicrographs of representative organs from *T. gondii*–infected mice treated with anti-IP-10 mAbs or control IgG. Hematoxylin and eosin stained formalin fixed sections of liver and spleen from *T. gondii*–infected mice treated with anti-IP-10 mAbs or control IgG. Liver of anti-IP-10-treated animal shows extensive hepatocellular necrosis with mixed inflammatory cells and visible tachyzoite proliferation (bar = 30 μM); inset, arrow shows intracellular tachyzoites (bar = 15 μM). Liver of control antibody–treated animal shows focal hepatocellular necrosis with mononuclear cell infiltrate but without visible tachyzoite replication. Center of a primary splenic follicle in anti-IP-10-treated animal shows necrosis and tachyzoite proliferation (bar = 50 μM); inset, arrow indicates intracellular tachyzoites (bar = 20 μM). The center of a primary splenic follicle of the control antibody animal showing largely preserved architecture with minimal necrosis and no visible tachyzoites. Similar treatment of uninfected mice with 1F11, 1B9, or control IgG had no effects on organ histopathology.

from the anti-IP-10-treated mice compared to control Ab–treated mice (Figures 7A and 7C). Therefore, although the proliferative response to mitogen was not affected by anti-IP-10 treatment of infected mice, the number of *T. gondii*–responsive $CD4^+$ and $CD8^+$ T cells in the spleen and liver was decreased compared to control Ab–treated infected mice. In contrast, the addition of anti-IP-10 mAb to splenocyte cultures established from infected untreated mice had no effect on antigen-induced proliferation (data not shown). These data suggest that anti-IP-10 treatment decreased the magnitude of the cell-mediated immune response in vivo. It is likely that the difference between these two groups reflects both diminished recruitment and subsequent expansion of antigen-specific $CD4^+$ and $CD8^+$ T cells in infected tissue.

Since cytolysis of infected cells by $CD8^+$ T cells also plays an important role in controlling *T. gondii* infection (Khan et al., 1994), we examined the ability of splenocytes isolated from infected anti-IP-10 and control Ab–treated mice to lyse *T. gondii*–infected macrophages in vitro. Significantly less *T. gondii*–specific cytolytic activity was seen for equivalent numbers of splenocytes isolated from anti-IP-10-treated infected mice compared to control Ab–treated infected mice ($p < 0.01$ at 40:1 E:T ratio and $p < 0.001$ at 20:1 E:T ratio for anti-IP-10 compared to control Ab group). To further analyze the differences in the number of antigen-specific cytotoxic T cells between IP-10-treated and -untreated mice, the precursor cytoloytic T lymphocyte (pCTL) assay was performed on spleen cells isolated from infected animals (Figure 7G). Splenocytes from both anti-IP-10- and control antibody–treated infected mice were cultured in a limiting dilution assay at day 12 post *T. gondii* infection and pCTL frequency of the cultures was determined. IP-10 neutralization of *T. gondii*–infected mice severely reduced the number of antigen-specific cytotoxic T cells. While the control antibody–treated infected mice showed pCTL frequency of $1/3.2 \times 10^3$, the anti-IP-10 mAb–treated infected mice had a pCTL frequency of $1/8.4 \times 10^4$, a 26-fold reduction. These findings are consistent with the hypothesis that anti-IP-10 treatment inhibited the recruitment of antigen-specific effector T cells into infected tissue. Interestingly, the production of *T. gondii*–specific IgG titers was unaffected by anti-IP-10

treatment of infected mice (Figure 7F), demonstrating that the B cell response was not grossly impaired.

Discussion

We have found that IP-10 is critical for survival following *T. gondii* infection and is essential for guiding antigen-specific $CD4^+$ and $CD8^+$ T cells into infected organs. *T. gondii* evokes a strong humoral and cellular immune response in the infected host (Decoster et al., 1988; Innes et al., 1995). Although antibody is generated against this pathogen, protective immunity is primarily dependent on the cellular immune response (Johnson, 1992). Immunity to *T. gondii* is strictly dependent on IFNγ production by $CD8^+$ T lymphocytes as well as NK and $CD4^+$ T cells (Suzuki et al., 1988; Deckert-Schluter et al., 1996). IFNγ has been thought to play its critical role by activating macrophage effector functions to control intracellular parasite replication (McCabe et al., 1984). Our findings suggest an additional role for IFNγ in host defense through the release of IFNγ-inducible chemokines, such as IP-10, in recruiting effector T cells to sites of infection. Thus, IP-10 represents a functional link between IFNγ production and T cell effector function required for parasite containment.

It has become increasingly clear that the chemokines orchestrate the trafficking of dendritic cells, T cells, and B cells necessary to generate an adaptive immune response (Cyster, 1999). Chemokines help bring together dendritic cells that have picked up antigen in the tissue into contact with naive T cells in secondary lymphoid organs. For example, the trafficking of antigen-loaded dendritic cells from the tissue into secondary lymphoid tissue appears to be under the control of the chemokine SLC (secondary lymphoid chemokine) and its receptor CCR7 (Forster et al., 1999; Gunn et al., 1999). SLC and CCR7 also play an important role in the recruitment of naive T cells into lymph nodes. The trafficking of B cells into lymph node germinal centers is regulated by the chemokine BLC (B lymphocyte chemokine) and its receptor CXCR5 (Forster et al., 1996). BLC and CXCR5 may also play an important role in movement of helper T cells once activated out of the T cell zone and into germinal centers to deliver their help to B cells (Ansel et al., 1999). Once activated, these effector T cells then home back to the site of infection and inflammation. The chemokines that control this trafficking of effector T cells out of lymphoid tissue and into inflamed tissue remain ill-defined. Our study provides strong evidence that IP-10 plays an important role in this process. We found that neutralization of IP-10 completely inhibited the influx of $CD4^+$ and $CD8^+$ cells into the spleen, liver, and lung. In addition, the marked increase in CXCR3 expression in the spleen, liver, and lung following infection was also blocked by neutralization of IP-10. Taken together, these results suggest that neutralization of IP-10 blocked the influx of activated $CD4^+$ and $CD8^+$ cells, which are known to express CXCR3.

The neutralization of IP-10 also had a dramatic effect on the magnitude of the effector response in the tissue. Antigen-specific proliferative responses were markedly reduced in $CD4^+$ and $CD8^+$ cells recovered from the spleens of infected anti-IP-10-treated mice compared to control Ab–treated infected mice. As was evident from our data (Figure 7), this was apparent even when equivalent numbers of purified $CD4^+$ and $CD8^+$ cells were analyzed. However, the response to mitogen was

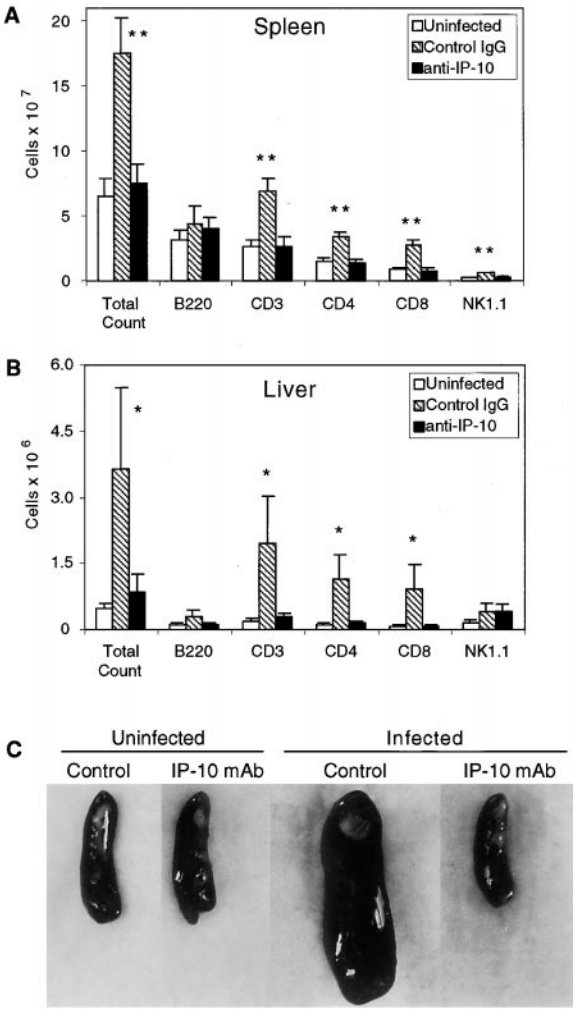

Figure 5. Neutralization of IP-10 In Vivo Inhibits the Massive Influx and Expansion of T Cells in *T. gondii*–Infected Organs

Immunophenotyping of splenocytes and hepatic lymphocytes from uninfected and *T. gondii*–infected mice. (A) Splenocytes and (B) hepatic lymphocytes from uninfected, infected/control IgG–treated, or infected/anti-IP-10-treated mice were quantified. Infected/control IgG–treated mice showed a significant increase in total splenocytes and hepatic lymphocytes compared to uninfected mice. Phenotyping revealed a significant increase in the $CD3^+$, $CD4^+$, and $CD8^+$ cell populations in both the spleen and the liver. In the spleen, $NK1.1^+$ cells were also significantly increased (asterisk, $p < 0.05$; double asterisk, $p < 0.005$). No change in $B220^+$ cells was seen in either the spleen or liver. In the infected/anti-IP-10–treated mice, no significant increase in either the total or individual lymphocyte subsets was seen, demonstrating that neutralization of IP-10 markedly decreased the number of T cells recovered from the liver and spleen compared to control IgG-treated infected mice (asterisk, $p < 0.05$; double asterisk, $p < 0.005$). Data are presented as mean cell counts (minimum 5 mice/group) ± SD from a representative of four independent experiments. (C) Representative spleens harvested from control Ab- and anti-IP-10-treated mice on day 12 following *T. gondii* infection. Uninfected mice also received either control Ab or anti-IP-10 Ab.

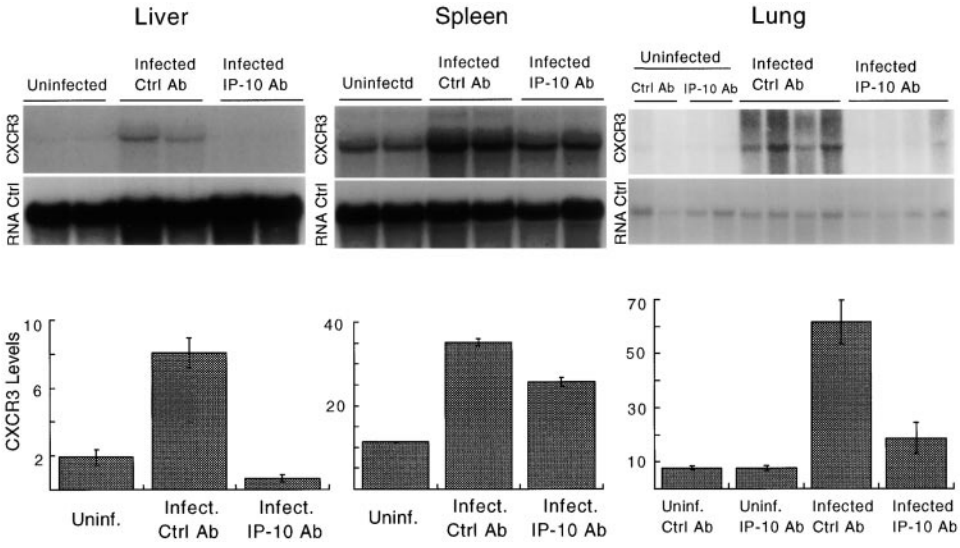

Figure 6. Neutralization of IP-10 In Vivo Blocks the Recruitment of CXCR3$^+$ T Cells into *T. gondii*–Infected Organs

Northern blot analysis of 15 μg of total RNA isolated from the liver, spleen, and lung of uninfected, uninfected/control Ab-treated, infected/control-Ab treated, and infected/anti-IP-10-treated mice on day 11 following oral infection with *T. gondii*. Each lane contains RNA from a different mouse. Blots were sequentially hybridizied with murine CXCR3 cDNA and GAPDH or actin as a control for RNA loading. Phosphorimagor analysis was used to quantify the CXCR3/(GAPDH or actin) mRNA signal for each sample. The inhibition of CXCR3 induction in infected organs is likely due to the ability of the anti-IP-10 mAb to inhibit the influx of CXCR3$^+$ T cells.

unaffected, suggesting that anti-IP-10 treatment affected antigen-specific proliferation and did not merely suppress proliferation non-specifically. This finding likely reflects the effects of anti-IP-10 treatment on the recruitment and subsequent expansion of antigen-specific cells in the spleen. We cannot exclude the possibility that anti-IP-10 treatment may somehow also inhibit antigen specific but not mitogen induced T cell proliferation. However, anti-IP-10 Abs had no effect on *T. gondii* induced proliferation in vitro on splenocytes isolated from infected untreated mice (data not shown). It was interesting to note that the IgG antibody response was unaffected by anti-IP-10 treatment. This suggests that sufficient antigen-specific CD4$^+$ helper cells were generated to produce a mature B cell response. Furthermore, it also suggests that these helper T cells were able to find their way to B cells to deliver their help. Thus, IP-10 does not appear to play a critical role in helper T cell and B cell trafficking within the secondary lymphoid tissues and germinal centers necessary to generate a humoral response. This would suggest that CXCR3$^+$ and CXCR5$^+$ may represent functionally distinct subpopulations of activated effector CD4$^+$ T cells. Since both of these receptors are upregulated on T cells following activation, perhaps IP-10/CXCR3 guide activated T cells into peripheral tissue while BLC/CXCR5 guide a different subpopulation into germinal centers.

Activated CD8$^+$ cytotoxic T cells must also be recruited into tissue where they play an important role in host defense against intracellular pathogens, including *T. gondii* (Khan et al., 1994; Denkers et al., 1997). Comparatively little is known about the molecular mechanisms controlling CTL trafficking in vivo; however, a recent study has suggested that chemokine receptors expressed on CTL may be involved. In a murine influenza model, the effective trafficking of CTL to sites of viral replication was a critical determinant of their ability to control viral replication and was associated with differential chemokine receptor expression, suggesting that this process is under the control of chemokines (Cerwenka et al., 1999). In *T. gondii* infection, CD8$^+$ T cells participate in host defense by both producing IFNγ and lysing infected cells (Khan et al., 1990; Hakim et al., 1991). Anti-IP-10 treatment reduced the cytolytic activity found in splenocyte cultures toward *T. gondii*–infected cells. This finding likely reflects the fact that there were fewer antigen-specific T cells in the spleen as IP-10 neutralization inhibited the influx of CD4$^+$ and CD8$^+$ T cells into infected spleens. This hypothesis was supported the observation that anti-IP-10-treated infected mice had a 26-fold reduction in the precursor CTL frequency compared to control Ab–treated infected mice. Thus, we believe our data is most consistent with the hypothesis that anti-IP-10 treatment inhibited the recruitment and subsequent expansion of antigen-specific CD8$^+$ T cells in the spleen. Again, we cannot exclude the possibly that IP-10 directly augments antigen-specific cytolytic function, although we have found no evidence that IP-10 directly effects CTL activity in vitro (A. D. L., unpublished data).

Our findings also dramatically demonstrate the nonredundant nature of the chemokine system in vivo, which is not appreciated by standard in vitro studies of chemotaxis. Despite the finding that the three known IFNγ-inducible CXCR3 ligands, IP-10, Mig, and I-TAC, are expressed in infected tissue, neutralization of IP-10 blocked effector T cell recruitment into tissue and markedly increased mortality. There are several possible explanations for these dramatic results. First, it is possible that IP-10 activates another yet unidentified receptor on effector T cells that it does not share with Mig and I-TAC. However, neutralization of IP-10 markedly inhibited the

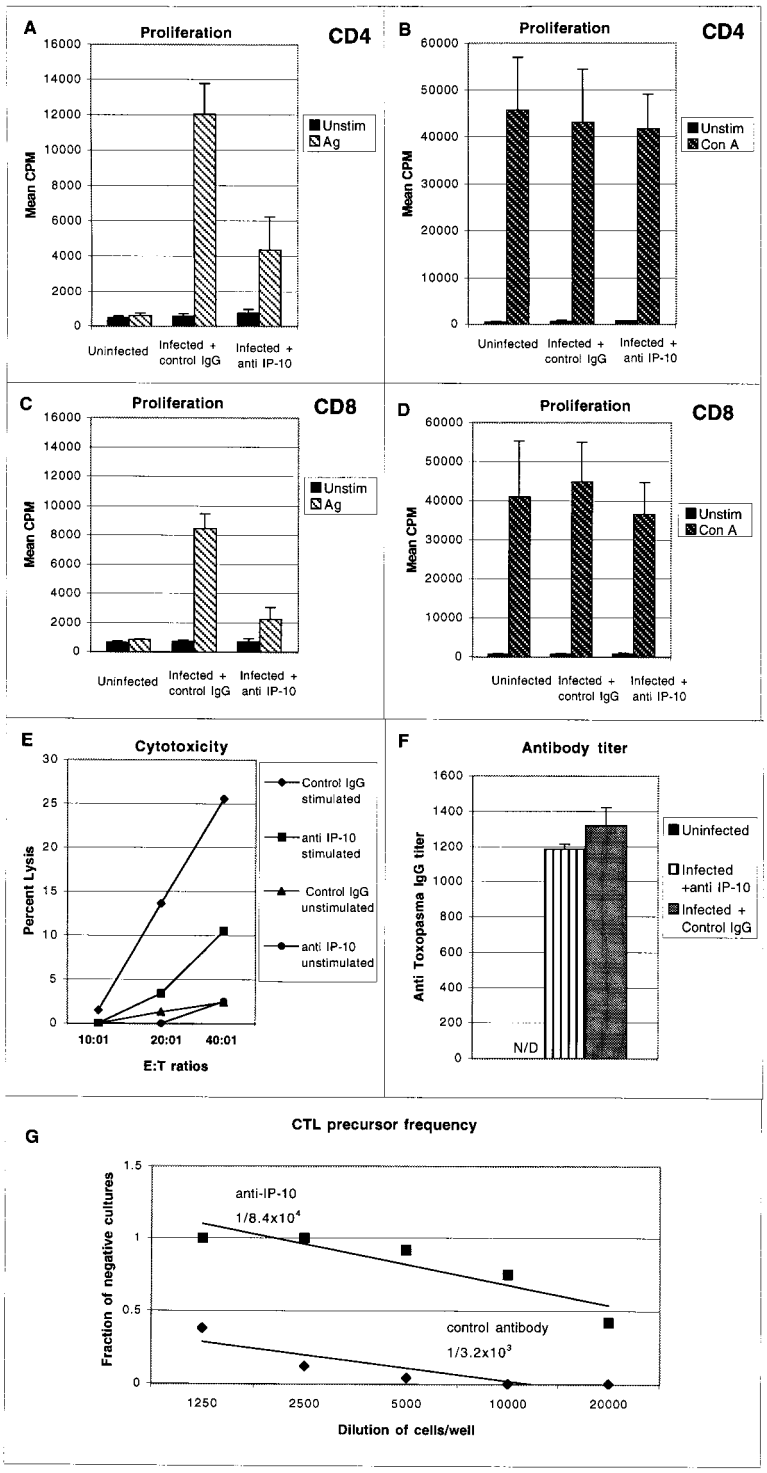

Figure 7. IP-10 Depletion Following *T. gondii* Infection Decreases Antigen-Specific T Cell Proliferation and Cytotoxicity

T. gondii–infected mice were treated with anti-IP-10 mAb or control IgG as described above. At days 10–12 post infection, splenocytes from anti-IP-10- and control antibody–treated animals were pooled (3 mice/group) and used for proliferation and cytotoxicity assays.

(A–D) Proliferation. CD4 (A and B) and CD8 (C and D) T cells were purified from splenocytes by positive magnetic selection (>95% pure by FACS analysis) and stimulated with either Con A (5 μg/ml) (B and D) or toxoplasma lysate antigen (TLA) (10 μg/ml) (A and C). After a 72 hr incubation period, ^{3}H-thymidine incorporation was measured over 8 hr to determine the rate of DNA synthesis. Data are presented as mean cpm ± SD and are representative of n = 3.

(E) Cytotoxicity. Spleen cells from anti-IP-10- and control IgG-treated mice were collected on days 10–12 post *T. gondii* infection and pooled (3 animals/group). Splenocytes were cultured for 5 days at 1×10^6 cells/well in the presence of 10 μg/ml of toxoplasma lysate antigen. Live cells were separated by Ficoll-Hypaque density centrifugation and incubated with ^{51}Cr-labeled macrophages infected with PLK strain of *T. gondii* or uninfected targets at various effector:target (E:T) ratios. Four hours after incubation, the cytolytic activity was determined by radioisotope release in the culture supernatant. Data are representative of n = 3, $p < 0.01$ for 40:1 E:T ratio and $p < 0.001$ for 20:1 E:T ratio, comparing anti-IP-10 to control Ab groups.

(F) Antibody titer. Serum from toxoplasma-infected mice treated with anti-IP-10 or control IgG was collected at day 12 post infection. Anti toxoplasma IgG titer was measured by ELISA (3 mice/group). The titers are expressed as mean ± SD. ND, not detected.

(G) CTL precursor frequency. Mice infected with *T. gondii* were treated with anti-IP-10 mAb or control Ab (n = 3/group). At day 12 post infection, splenocytes were isolated, pooled, and cultured in a limiting dilution assay as described in Experimental Procedures. After one week in culture, the pCTL frequency of the cultured cells was determined. Spleen cells from anti-IP-10-treated infected mice had 26-fold less precursor cytotoxic T lymphocytes as compared to control Ab-treated infected mice ($1/8.4 \times 10^4$ versus $1/3.2 \times 10^3$).

influx of CXCR3$^+$ T cells, suggesting that in this model, IP-10 plays a dominant role in the recruitment and/or retention of CXCR3$^+$ T cells in infected tissue. Second, it is possible that the timing and pattern of chemokine expression in tissue is a critical determinant dictating a given chemokine's function in a given infectious or inflammatory disease/model. Thus, the different roles of IP-10, Mig, and I-TAC in vivo may be due to the differential expression of these chemokins in situ. For example, in addition to IFNγ, IP-10 expression is induced by LPS and IFNα/β, while Mig induction is restricted to IFNγ. The expression of IP-10, Mig, and I-TAC is controlled by unique promotors with different regulatory elements: IP-10 induction is dependent on two NF-κB sites and one IRSE site (Ohmori and Hamilton, 1993), while Mig induction is dependent on a tandem

GAS element (Wong et al., 1994). Following *T. gondii* infection, we found that IP-10 was induced at earlier time points compared to Mig and I-TAC. This differential induction may reflect the absolute dependence of Mig but not IP-10 on IFNγ. In fact, we and others have seen that following infection of IFNγ$^{-/-}$ mice with *T. gondii*, IP-10 expression is reduced but not eliminated, whereas Mig expression is completely eliminated (Amichay et al., 1996). Thus, perhaps the early expression of IP-10 is induced by products of *T. gondii* that activate the innate immune system resulting in NF-κB activation. This early IP-10 expression then recruits activated Th1 cells that release IFNγ and amplify the local Th1 response. Alternatively, IP-10, Mig, and I-TAC may not only be expressed at different times but also in different places within a tissue leading to functional differences. We have recently found evidence that this can occur in situ in a human inflammatory disease. In human atherosclerotic lesions, we have found that IP-10 is expressed in endothelial cells, macrophages, and smooth muscle cells, while the expression of Mig and I-TAC was seen only in endothelial cells and macrophages and not in smooth muscle cells, an important component of the vessel (Mach et al., 1999). Third, it is also possible that IP-10, Mig, and I-TAC differentially activate CXCR3, resulting in unique biological functions in vivo. This is an intriguing hypothesis that warrants further evaluation.

It was noted that at later time points following infection, multiple chemokines were found to be highly expressed in infected tissues. This is in contrast to early time points, where there was the differential expression of certain chemokines, such as IP-10. In the *T. gondii* model, this early expression of IP-10 plays a critical role in establishing an effective host response to infection. The expression of multiple chemokines at later time points likely reflects the recruitment of activated effector T cells into tissues that elaborate and induce a "second wave" of chemokine expression. This may be relevant to the analysis of chemokine expression in human disease tissue where typically lesional tissue is analyzed at a single time point, often late in the disease. Thus, our data suggests that relevant chemokines that help establish the nature of the immune response in a tissue may be those that are induced at early time points following the infectious or inflammatory stimulus.

Finally, the broader implications of these data are that IP-10 may play an important role in the recruitment of activated effector T cells into tissues in other Th1-type inflammatory diseases where it has been shown to be highly expressed, such as psoriasis (Gottlieb et al., 1988), sarcoidosis (Agostini et al., 1998), multiple sclerosis (Balashov et al., 1999; Sorensen et al., 1999), and atherosclerosis (Mach et al., 1999). Further investigations will establish whether IP-10 is functionally important in these inflammatory conditions and whether this chemokine and/or its receptor will be targets for therapeutic intervention.

Experimental Procedures

Mice and Infection

Five- to six-week-old C57BL/6 were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were challenged perorally with the 76K strain of *T. gondii* (kindly provided by Dr. Daniel Bout, Tours, France). This strain is maintained by continuous oral passage of cysts. Mice were infected perorally with 10–15 cysts. For in vivo antibody experiments, groups of 5–10 mice were injected intraperitoneally every other day with 100 μg of either hamster anti-IP-10 mAb in 200 μl of PBS or 100 μg of hamster IgG control Ab (Jackson ImmunoResearch Laboratories, West Grove, PA).

RNase Protection and Northern Analysis

RNA was prepared using Trizol (GIBCO–BRL, Grand Island, NY) or lysis in guanidium hydrochloride followed by pelleting through a $CsCl_2$ gradient. For the RNase protection analysis, 10 μg of RNA from each sample was used, in duplicate. The probe set was kindly provided by Iain Campbell (Scrips, La Jolla, CA) (Asensio and Campbell, 1997). The analysis was performed using a Riboquant RPA kit (PharMingen, San Diego, CA). For quantification, the autoradiographs were scanned and the band density was measured using NIH image 1.62 software, and the intensity of each band was normalized to the intensity of the control rpL32 for each sample. For Northern analysis, 15 μg total RNA was electrophoresed on a 1.2% agarose formaldehyde gel and then capillary transferred to a GeneScreen membrane (NEN Life Science Products, Boston, MA). Following prehybridization (50% formamide, 1% SDS, 4× SSC, 4× Denhardt's solution, 0.8% glycine, and 0.17 mg/ml denatured salmon sperm DNA) at 42°C, blots were hybridized at 42°C in 50% formamide, 10% dextran, 1% SDS, 5× SSC, 1× Denhardt's solution, and 0.17 mg/ml denatured salmon sperm DNA with 1×10^6 cpm/ml [α-^{32}P]dCTP cDNA probe prepared by nick translation. The following fragments were used as probes: a 380 bp coding fragment of the murine IP-10 (crg) cDNA, a 1.4 kb full-length fragment of the murine Mig cDNA (kindly provided by J. Farber, NIH), a 829 bp fragment of the murine I-TAC cDNA (kindly provided by G. Werner-Felmayer) (Meyer et al., 2000), and a 1 kb fragment of the CXCR3 cDNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (kindly provided by M. Prystowsky) and β-actin cDNA probes were used as controls for RNA loading. Signal quantitation was determined using a phosphoimager (Molecular Imager System, Bio-Rad, Hercules, Ca), and values for each sample were normalized based on the GAPDH or actin signal.

Generation and Characterization of Anti-IP-10 mAbs

Anti-murine IP-10 mAbs were generated by immunizing Armenian hamsters with recombinant *E. coli*–produced murine IP-10 in complete Fruend's adjuvant followed by three booster immunizations of IP-10 in incomplete Freund's adjuvant. Splenocytes were fused to P3X63 myeloma cells using a standard polyethylene glycol 1500 protocol. A solid phase direct enzyme-linked immunosorbent assay (ELISA) was used to identify positive clones, which were then subcloned three times by limiting dilution. mAbs were tested for their specificity using available purified mouse chemokines, including Mig, MIP-1α, MIP-1β, SDF-1, KC, TCA-3, RANTES, eotaxin, MCP-1, MCP-3, and MCP-5 in a direct ELISA and immunoblot assay. 1F11 and 1B9, two mAbs specific for murine IP-10, were adapted to serum-free medium (50% DMEM and 50% hybridoma serum-free (GIBCO) and grown in a CELLMAX cartridge (CELLCO). 1F11 and 1B9 IgG were purified from the serum-free medium by Protein A affinity chromatography (Pharmacia, Piscataway, NJ). These purified mAbs contained <10 ng of endotoxin per milligram of IgG as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). To test the ability of these mAbs to inhibit IP-10-induced chemotaxis, a murine CXCR3 stably transfected 300-19 B cell line was generated (Luster et al., 1998) and used as a responding cell line in chemotaxis and calcium flux assays. Chemotaxis was performed in a modified Boyden chamber as described (Luster et al., 1998), and calcium flux was measured in a PTI DeltaRam Flourimeter.

Quantitation of Parasite DNA

DNA was extracted from tissues using the Qiamp tissue kit (Qiagen, Chatsworth, CA), and 400 ng of each sample was analyzed. Amplification of parasite DNA was performed using primers specific for the toxoplasma B1 gene (5′-GGAACTGCATCCGTTCATGAG-3′ and 5′-TCTTTAAAGCTTCGTGGTC-3′), a 35-fold repetitive sequence found in all known parasite strains (Burg et al., 1989). A 134 bp

competitive internal standard containing the same primer template sequences as the 194 bp B1 PCR fragment was generated and used as an internal standard for competitive PCR as described (Kirists et al., 2000). Amplification of the 194 base pair segment of the B1 gene and the 134 base pair segment of the internal standard was performed in a 50 μl reaction mix containing 1.25 units of Amplitaq DNA polymerase, 1× buffer (Perkin Elmer), 0.2 mM each of dGTP, dATP, dTTP, and dCTP, and 0.4 μM (each) B1 primers. For each reaction, a known amount of DNA from the tissues was amplified with varying amounts of the internal standard. To determine the parasite load for the infected tissues, PCR was performed with the same conditions using a known number of parasites. The level of the internal control was calculated per parasite (Kirists et al., 2000).

Phenotypic Analysis

Single cell suspensions of splenocytes were prepared using standard procedures, and hepatic lymphocytes were isolated using a modification of published methods (Watanabe et al., 1992). In brief, livers were perfused with sterile PBS prior to excision to remove blood elements. Excised livers were minced and passed through a mesh screen. The cells were washed with PBS and subjected to density gradient centrifugation on Lympholyte-M (Accurate Chemical and Scientific, Westbury, NY) to isolate the lymphocyte population. The splenocytes and hepatic lymphocytes were suspended in PBS 10% FCS and counted in a hemocytometer prior to staining with the following fluorochrome-conjugated antibodies: anti-B220 PE, anti-CD3 FITC, anti-CD8 FITC, and anti-NK1.1 PE (all from PharMingen) and anti-CD4 Red613 (GIBCO–BRL). Two- and three-color flow cytometry was performed on a FACscan cytometer (Becton Dickinson, San Jose, CA), and the data was analyzed using Lysys software (Hewlett-Packard, Palo Alto, CA).

Proliferation Assay

$CD4^+$ and $CD8^+$ T cells were purified from splenocytes using positive selection with microbeads (Milltenyl Biotec, Auburn, CA) to a purity of >95% as determined by flow cytometry. Purified cells were cultured in 96-well plates at 2×10^5 cells/well in 200 μl. The cells were stimulated with either Con A (5 μg/ml; Sigma Chemical) or toxoplasma lysate antigen (TLA; 10 μg/ml) (Khan and Kasper, 1996) in the presence of 1×10^5 irradiated feeder cells (3000 rads) obtained from syngeneic mice. After 72 hr incubation at 37°C in 5% CO_2, cells were pulsed with ^{3}H-thymidine (0.5 uci/well) for 8 hr to determine rate of DNA synthesis. Pulsed splenocytes were harvested on a glass filter, dried, and radioactive thymidine corporation measured by liquid scintillation.

Cytotoxicity Assay

Cytotoxic T lymphocyte (CTL) assays were performed according to standard procedures (Ely et al., 1999). Briefly, mouse peritoneal macrophages were obtained by lavage, two days after i.p. inoculation with 1 ml of thioglycollate. The macrophages were washed three times in PBS and dispensed at a concentration of 3×10^4 cells/well in U-bottom tissue culture plates in medium. Macrophages were incubated overnight and the next morning labeled with ^{51}Cr (0.5 μci/well, New England Nuclear Research Products, Boston, MA) for 3 hr at 37°C. After washing several times in PBS (or until supernatant contained <500 cpm), the macrophages were infected with freshly isolated cell culture grown tachyzoites of PLK strain at a concentration of 5×10^4 parasites/well and incubated overnight. The next morning, spontaneous lysis caused by overnight parasite infection was measured, and wells exhibiting >500 cpm in the supernatants were excluded from the experiment. Macrophages were washed three times in PBS and incubated with splenocytes at various effector:target ratios in a final volume of 200 μl of culture medium. Following the addition of spleen cells, the microtiter plates were centrifuged at 200 × g for 3 min and incubated at 37°C for 3 hr. One hundred microliters of supernatant from each sample was removed and assayed for released counts per minute (cpm) by scintillation counting. The percent lysis was calculated as follows: (mean cpm of test sample − mean cpm of spontaneous release)/(mean cpm of maximal release − mean cpm of spontaneous release) × 100.

Precursor Cytotoxic T Lymphocyte Frequency

To quantitate the frequency of precursor cytotoxic T lymphocytes (pCTL), spleen cells from infected anti-IP-10- and control Ab–treated mice were cultured by limiting dilution in 96-well round bottom plates at day 12 post infection. The cells were grown in RPMI 1640 medium containing appropriate growth factors, including 15 units/ml of recombinant IL-2 (R&D Chemicals, Minneapolis, MN) irradiated tachyzoites of PLK strain and feeder cells. Cells were diluted from 20,000 to 1,250 cells/well. Control wells contained only irradiated parasites and feeder cells. Syngenic splenocytes irradiated at 3,000 rads were used as feeder cells. After one week, the cells were harvested and incubated with ^{51}Cr-labeled parasite-infected and -uninfected macrophages. The macrophages were collected and labeled as described above. The wells were considered to be positive for lytic activity if total cpm released by effector cells plus target cells was greater than 3× SD above control wells (mean cpm released by the target cells incubated with irradiated parasites and feeder cells). The pCTL frequency was calculated according to the standard formula as described (Taswell, 1981).

Antibody Titer

Antitoxoplasma antibody titer was measured by ELISA as described (Khan et al., 1991). Purified parasites (5×10^4/ well) were placed in microdilution plates, dried overnight, and blocked with 5% fetal bovine serum PBS. Antisera were incubated for 2 hr at 37°C, and the plates were washed in 0.05% Tween 20 PBS. Antibody binding was identified with a peroxidase-labeled goat anti-mouse IgG (Sigma Chemicals).

Statistical Analysis

Results of experimental studies are reported as mean ± standard deviation. Differences were analyzed using the Student's t test as described (Neter et al., 1985). A p value < 0.05 was regarded as significant.

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